

TITLE:            PRODUCTION OF SOMATIC  
                 MOSAICISM IN MAMMALS USING A  
                 RECOMBINATORIAL SUBSTRATE

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PRODUCTION OF SOMATIC MOSAICISM IN MAMMALS  
USING A RECOMBINATORIAL SUBSTRATE

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FIELD OF THE INVENTION

15           The present invention relates to DNA molecules  
encoding recombinatorial substrates which can be activated  
to effect a gain or loss of function of genes in somatic  
and/or germ cells of a mammal, methods for creating the  
recombinatorial substrates, and methods for activating the  
20 recombinatorial substrates in mammals.

BACKGROUND OF THE INVENTION

          Creating focal genetic modifications in an intact  
animal is a powerful approach for studying the cellular  
25 interactions that underlie the development and function of  
tissues and organs. This method has been used to great  
advantage in *Drosophila*, facilitating the study of  
developmental questions relating to the autonomy of gene  
actions, restriction of cell fate and growth pattern of  
30 specific tissues. The approach involves the generation of  
genetic mosaics, tissues in which some cells differ from  
their neighbors by a single mutation, effecting either a  
gain or loss of function phenotype. Through the analysis of  
mutant and wild-type cells within mosaics patches, it is  
35 possible to draw inferences about interacting cells and in  
some cases the molecules and pathways subserving cellular  
communication.

          The application of mosaic analysis to the study of  
nervous system function has the potential to yield a wealth  
40 of information because it should allow for an assessment of

the function of particular gene products within individual cells that are part of a network. Thus, in tissues such as the nervous system where functional information resides not only in the nature and number of its constituent cells, but  
5 also in the manner in which they connect and temporally interact, it is essential that strategies be employed that neither unintentionally change the network nor eliminate some of the cellular constituents. Overall, the ideal strategy should permit stable genetic modification with  
10 precise temporal and spatial control.

Implicit in the use of genetic mosaic analysis is the ability to distinguish mutant from normal cells by the use of markers. The preferred marker is one which is gratuitous thus causing no cell damage, cell-autonomous so  
15 that cellular level resolution of mosaicism can be reliably scored, and having a short half-life to improve temporal analysis of the tissue after genetic modification.

Genetic mosaics have been generated in *Drosophila* by induction of mitotic recombination (reviewed in  
20 Ashburner, M., Drosophila: A Laboratory Handbook, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989), which is hereby incorporated by reference). Typically, radiation is used to induce DNA damage. In cells that have just completed DNA synthesis but have not yet  
25 divided, repair of the damage leads infrequently to homologous chromosome exchange. If the homologous chromosomes are appropriately marked, the resultant recombination event can be scored in daughter cells. More recently, high-frequency homologous chromosome exchange has  
30 been achieved by the use of the yeast FLP/FRT system (Golic K., Science, 252, 958 (1991); Golic, K. and Lindquist, S. Cell, 59, 499 (1989); Harrison, D. and Perrimon, N., Curr. Biol., 3, 424 (1993); Xu, T. and Rubin, G., Development, 117, 1223 (1993), which are hereby  
35 incorporated by reference). The FLP gene product is a site specific recombinase that catalyzes recombination at FRT target DNA elements. Expression in flies of FLP induces

recombination between FRT elements on homologous chromosomes (Harrison, D. and Perrimon, N., Curr. Biol., 3, 424 (1993); Xu, T. and Rubin, G., Development, 117, 1223 (1993), which are hereby incorporated by reference). Inducible control of FLP by a heat shock promoter has been used in flies to grade the extent of recombination, thus modulating the extent of genetic mosaicism (Xu, T. and Rubin, G., Development, 117, 1223 (1993), which is hereby incorporated by reference).

Genetic mosaics in flies have also been generated by inducing intramolecular chromosomal recombination with the FLP/FRT system. In one example of this strategy, a transgene is constructed such that it is inactivated by the insertion of a DNA sequence encoding a stop codon flanked by FRT sites. After induction of FLP the inactivating DNA cassette is 'flipped-out' allowing for the transcription of an mRNA that yields a translatable gene product (Struhl, G., Fitzgerald, K. and Greenwald, I., Cell, 73, 1323 (1993), which is hereby incorporated by reference). This approach could be used to generate gains or loss of function at any transcriptionally active transgene integration site or at a specific gene location targeted by homologous recombination.

Although the use of the FLP/FRT system in mammalian cells has been reported (O'Gorman, S., Fox, D. and Wahl, G., Science, 251, 1351 (1991), which is hereby incorporated by reference), a different recombination system, cre/loxP, has received wider attention and apparently greater success. The cre recombinase is bacteriophage P1-derived, and it interacts with its target site, loxP, a 34 bp element to produce site specific recombination (Sauer, B. and Henderson, N., Proc. Natl. Acad. Sci. USA 85, 5166 (1988); Sternberg, N. and Hamilton, D., J. Mol. Biol., 150, 467 (1981), which are hereby incorporated by reference). Using a binary approach in transgenic animals, investigators constructed and introduced separately into the germline of mice two different transgenes; the first, a strong promoter driving cre, and the second a recombinatorial substrate which

contained two loxP sites (Lasko, M., et al., Proc. Natl. Acad. Sci. USA, 89, 6232 (1992); Orban, P., Chiu, D. and Marth, J., Proc. Natl. Acad. Sci. USA, 89, 6861 (1992),

which are hereby incorporated by reference). Crossing the two transgenic lines gave rise to compound heterozygotes in which recombination occurred in a highly efficient manner.

The cre/loxP system has also been applied in embryonic stem (ES) cells to create deleted alleles by targeting a homologous locus with a construct that contains loxP

elements flanking the region to be excised (Gu, H., Zou, Y.-R. and Rajewsky, K., Cell, 73, 1155 (1993), which is hereby incorporated by reference). With this approach the transient expression of cre produced a significant frequency of recombination events (Gu, H., Zou, Y.-R. and

Rajewsky, K., Cell, 73, 1155 (1993), which is hereby incorporated by reference). Overall, the use of recombination systems in mice appears to satisfy the need for the efficient creation of stable genetic mosaics.

However, the approach is significantly limited by constraints imposed by the characteristics of the promoter chosen to express the recombinase. Since every cell that expresses the recombinase will likely suffer a recombination event, it is difficult to use the system to generate mosaic tissues.

A need exists for a system to produce stable genetic mosaics where precise temporal and spatial control of gene expression can be obtained.

#### SUMMARY OF THE INVENTION

The present invention relates to DNA molecules having recombinatorial substrates which can be activated to effect a gain or loss of function of a gene within the DNA molecule.

One aspect of the present invention relates to a DNA molecule encoding a recombinatorial substrate having (1) a promoter element capable of promoting transcription of genes in the recombinatorial substrate, (2) a gene whose

expression is to be controlled, which is positioned 3' to the promoter element to facilitate its transcription, (3) a terminator positioned 3' to the promoter element and 5' to the gene whose expression is to be controlled to prevent  
5 transcription of the genes 3' to the terminator, and (4) two recombination sites located 3' and 5' to the terminator. The recombinatorial substrate is arranged such that treatment of the DNA molecule with a recombinase specific to the recombination sites removes the terminator from the DNA  
10 molecule, thus activating the recombinatorial substrate and permitting transcription of the gene whose expression is to be controlled.

Another aspect of the invention is a method of producing a transgenic mammal whose somatic and germ cells  
15 contain the recombinatorial substrate which, when treated by recombinase, transcription of the gene whose expression is to be controlled occurs. This recombinatorial substrate is introduced into an embryo which is then transplanted into a pseudopregnant mammal. The resulting transplanted embryo is  
20 allowed to develop to term. A mammal which carries the recombinatorial substrate is identified.

Another aspect of the invention is a method of activating the recombinatorial substrate which, when treated by recombinase, permits transcription of the gene whose  
25 expression is to be controlled. A recombinase is introduced into a transgenic mammal, causing this recombinatorial substrate to excise the terminator between the recombination sites. A mammal containing an activated recombinatorial substrate is then identified.

30 Yet another aspect of the invention is a DNA molecule encoding a recombinatorial substrate having (1) a promoter element capable of promoting transcription of genes in the recombinatorial substrate, (2) a gene whose expression is to be controlled, the gene being positioned 3'  
35 to the promoter element to facilitate its transcription, and (3) two recombination sites located 3' and 5' to the gene whose expression is to be controlled. The recombinatorial

substrate is arranged such that treatment of the DNA molecule with a recombinase specific to the recombination sites removes the gene whose expression is to be controlled from the DNA molecule, thereby activating the

5 recombinatorial substrate and resulting in the loss of function of the gene whose expression is to be controlled.

A further aspect of the invention is a method of producing a transgenic mammal whose somatic and germ cells contain a recombinatorial substrate which, when treated by  
10 recombinase, permits excision of the gene whose expression is to be controlled. This recombinatorial substrate is introduced into an embryonal stem cell which is integrated into a blastocyst which is then transplanted into a pseudopregnant mammal. The resulting transplanted embryo is  
15 allowed to develop to term. A mammal which carries the recombinatorial substrate is identified.

Another aspect of the invention is a method of activating the recombinatorial substrate which, when treated by recombinase, permits excision of the gene whose  
20 expression is to be controlled. A transgenic mammal, carrying this recombinatorial substrate is treated with a recombinase to excise the gene whose expression is to be controlled thereby activating the recombinatorial substrate and resulting in a loss of function of the gene whose  
25 expression is to be controlled. A mammal which contains an activated recombinatorial substrate is then identified.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 summarizes the structure and use of  
30 recombinatorial substrates. Figure 1A is a diagram of a recombinatorial substrate which activates the gene to be controlled. Figure 1B depicts a recombinatorial which when activated results in the loss of function of the gene to be controlled.

35 Figure 2 shows the NGF-XAT construct and recombination driven excision. Figure 2A is a diagram of NGF-XAT before and after cre-mediated recombination. NGF-

XAT was transformed into *E. coli* that either did not express (Figure 2B and 2C, lanes 1-9) or did express cre recombinase (lanes 11-22). Plasmid DNA was isolated, digested and hybridized to the probes indicated in Figure 2A.

5           Figure 3 shows northern blot analysis of brain mRNA from NGF-XAT transgenic lines. Figure 3A is a diagram of the NGF-XAT recombinatorial substrate and the expected short mRNA product. Northern hybridization of PolyA RNA with an NGF-XAT probe isolated from adult transgenic mice  
10       that is probed with an NGF-XAT probe. Figure 3B demonstrates expression of the short NGF-XAT mRNA in two of the lines of transgenic mice with the NGF-XAT construct.

          Figure 4 is an *in situ* hybridization performed on 12  $\mu$ m coronal sections using the Digoxigenin labeled  
15       oligonucleotide probes (45 mer's) complementary to hGH sequences contained within the NGF-XAT recombinatorial substrate. Figure 4A uses an antisense probe and low power view encompassing the cortex, hippocampus and part of the thalamus. The CA1 and CA3 pyramidal and dentate gyrus  
20       regions of the hippocampus are labeled. Figure 4B uses an antisense probe with high power view (boxed area in 4A) of dentate gyrus and pyramidal cell labeling. Figure 4C uses a sense probe and low power view of encompassing cortex, hippocampus and part of the thalamus.

25           Figure 5 shows HSVcre delivery *in vivo* to NGF-XAT mice. Figure 5A is a diagram of HSVcre. Figure 5B is a western blot of protein extracts isolated from NIH3T3 cells infected with HSVcre. Figure 5C is a depiction of the NGF-XAT construct before and after recombination showing the  
30       location of the PCR primers. Figure 5D is a PCR-Southern analysis to monitor CRE recombination *in vivo*. Figure 5E is a nested PCR analysis to monitor recombination.

          Figures 6A-6C are the X-gal histochemistry results to show localization of the vector expression. Figure 6D  
35       shows the marked increase in NGF immunoreactivity in the virus injection region. Figure 6B is the histochemistry of



the injection region using HSVlac. Figures 6A and 6C are regions anterior and posterior to the injection site.

Figure 7 is a bar graph depicting the NGF content from HSVcrelac and HSVlac injected animals.

5                Figure 8 is a graph of the horizontal and vertical activity counts for each five minute interval for HSVcrelac and HSVlac mice.

#### DETAILED DESCRIPTION OF THE INVENTION

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The study of gene product function in a varied organ systems has been facilitated by somatic mosaic analysis, whereby stable somatic mutations that result in either gain or loss of function are created and which allow  
15 for analysis of phenotypes among groups of cells genotypically identical except for the generated mutation. Ashburner, M., Drosophila: A laboratory handbook (1989), which is hereby incorporated by reference. To develop a related approach in mammals allowing for the production of  
20 focal and stable genetic changes in the postnatal animal, this invention provides a combined binary somatic and germline gene transfer strategy.

One aspect of the present invention relates to a recombinatorial substrate and its use, where activation of  
25 the substrate causes a gain of function by permitting expression of a gene in the substrate. This aspect of the present invention utilizes a DNA molecule encoding a recombinatorial substrate having (1) a promoter element capable of promoting transcription of genes in the  
30 recombinatorial substrate, (2) a gene whose expression is to be controlled, which is positioned 3' to the promoter element to facilitate its transcription, (3) a terminator positioned 3' to the promoter and 5' to the gene whose expression is to be controlled to prevent transcription of  
35 genes 3' to the terminator, and (4) two recombination sites located 3' and 5' to the terminator. The recombinatorial substrate is arranged such that treatment of the DNA

molecule with a recombinase specific to the recombination sites removes the terminator from the DNA molecule, thus activating the recombinatorial substrate and permitting transcription of said gene whose expression is to be controlled.

Figure 1A depicts an example of such a DNA molecule before and after activation. The recombinatorial substrate prior to recombination has a promoter. Following the promoter, a stop, i.e. terminator, is located between two recombinase specific recombination sites. In the example in Figure 1A the recombinatorial sites are LOX sites. The gene to be controlled is represented by the Exons. A reporter gene is located downstream followed by a polyA region, a 3' flanking region to stabilize the transcript. The recombinatorial substrate is then activated by a recombinase. In the example depicted, the recombinase is introduced on a herpes simplex virus. The activated recombinatorial substrate is shown, where the region between the LOX sites has been deleted. This results in the expression of the gene whose expression is to be controlled and the reporter gene.

Yet another aspect of the invention relates to a recombinatorial substrate and its use where activation of the substrates causes a loss of function by precluding expression of the gene whose expression is to be controlled by activating recombination sites surrounding the gene thereby deleting the gene. A further aspect of the present invention utilizes a DNA molecule encoding a recombinatorial substrate having (1) a promoter element capable of promoting transcription of genes in the recombinatorial substrate, (2) a gene whose expression is to be controlled, the gene being positioned 3' to the promoter element to facilitate its transcription, and (3) two recombination sites located 3' and 5' to the gene whose expression is to be controlled. The recombinatorial substrate is arranged such that treatment of the DNA molecule with a recombinase specific to the recombination sites removes the gene whose expression is

to be controlled from the DNA molecule, thereby activating the recombinatorial substrate and resulting in the loss of function of the gene whose expression is to be controlled.

Figure 1B depicts a recombinatorial substrate  
5 which when activated results in the loss of function of the gene whose expression is to be controlled. The gene in the germline has a promoter, followed by the exons coding for the gene product, and a 3' flanking region. The recombinatorial substrate is constructed with recombination  
10 sites on each side of the gene such that when recombination occurs the gene is inactivated. In the example depicted in Figure 1B, the recombinatorial substrate also has a terminator, or stop, located between the recombination sites. A reporter gene is located 3' to the terminator and  
15 when the substrate is activated the reporter gene is transcriptionally activated allowing for facilitated identification of activated recombinatorial substrates. Figure 1B also identifies the recombination sites as LOX sites, a preferred embodiment of the invention. The  
20 recombinatorial substrate is introduced into the germline of the organism through homologous recombination as depicted in Figure 1B. The recombinatorial substrate can then be activated by introducing recombinase into the cells of the mammal. In Figure 1B, the recombinase is introduced via a  
25 herpes simplex virus. The activated substrate is shown at the bottom of Figure 1B after recombination. The region between the recombination sites is deleted leaving only one copy of the recombination site. The loss of the gene between the recombination sites results in the loss of  
30 function of that gene in the mammalian cells where the substrate is activated.

To facilitate the identification of cells which carry an activated recombinatorial substrate, a reporter gene can be placed 3' to the terminator and the  
35 recombination sites. The reporter gene produces either an RNA or peptide which can be readily detected. Preferred reporter genes include the genes encoding LacZ,

chloramphenicol acetyl transferase, luciferase, the green fluorescent protein, human alkaline phosphatase, hygromycin unresistor gene; and neomycin phospho transferase.

5 The DNA molecule may also consist of a 3' flanking region which will stabilize the transcript made by the molecule and terminate transcription coming from the molecule, located 3' to the gene to be expressed. (Moreira, A., Wollerton, M., Monks, J., Proudfoot, N.J., EMBO J., 14, 3809 (1995), which is hereby incorporated by reference) The  
10 3' flanking region contains a transcription terminator and stabilizing elements such as a polyA region. Therefore, the 3' flanking region will be located where the transcript will terminate. The preferred 3' flanking sequences include the 3' flanking regions from the genes encoding  $\beta$ -galactosidase,  
15 SV40,  $\beta$ -globin,  $\alpha$ -globin, and human growth hormone. The most preferred 3' flanking sequence is a 3' flanking sequence from a human growth hormone gene.

The preferred promoter elements include promoters from the genes encoding: myosin heavy chain  $\alpha$ , myosin heavy  
20 chain  $\beta$ , insulin, somatostatin, glucagon, growth associated protein 43 kDa, superior cervical ganglion clone 10, neurofilament-L, neurofilament-M, neurofilament-H, glial bifilary protein, P0, myelin associated glycoprotein, myelin basic protein, calcitonin-gene related peptide, and a neuron  
25 specific enolase. The most preferred promoter element is a neuron specific enolase promoter.

The preferred recombinase sites include FRT and LoxP sites. The most preferred recombinase sites are LoxP sites.

30 The preferred terminators include transcription terminators for gastrin, C2 complement, and  $\beta$ -globin. The most preferred terminator is a  $\beta$ -globin transcription terminator.

35 This approach has broad application to the regulation of numerous genes. In particular this application is useful for creating and studying discrete modifications in genes where the spacial and temporal

expression of the gene is important. Therefore, the genes to be controlled will include genes expressing regulatory factors, signal transducers, and developmental factors.

Although any gene may be used, the preferred genes  
5 whose expression is to be controlled includes genes  
expressing hormones, hormone receptors, neurotransmitters,  
neurotrophic factors, neurotrophic factor receptors,  
neuronal peptides, cell signaling molecules, and receptors  
for any of these peptides. The most preferred genes whose  
10 expression is to be controlled includes genes expressing  
neuronal growth factors.

The present invention also relates to any vectors  
which contain the recombinatorial substrates. Vectors  
include viral and plasmid vectors. Generally, this involves  
15 inserting the selected DNA molecule into an expression  
system to which that DNA molecule is heterologous (i.e. not  
normally present). The heterologous DNA molecule is  
inserted into the expression system or vector in proper  
orientation and correct reading frame. The vector contains  
20 the necessary elements for the transcription and translation  
of the inserted protein-coding sequences.

U.S. Patent No. 4,237,224 to Cohen and Boyer,  
which is hereby incorporated by reference, describes the  
production of expression systems in the form of recombinant  
25 plasmids using restriction enzyme cleavage and ligation with  
DNA ligase. These recombinant plasmids are then introduced  
by means of transformation and replicated in unicellular  
cultures including procaryotic organisms and eucaryotic  
cells grown in tissue culture.

30 Recombinant genes may also be introduced into  
viruses, such as vaccina virus. Recombinant viruses can be  
generated by transfection of plasmids into cells infected  
with virus.

Suitable vectors include, but are not limited to,  
35 the following viral vectors such as lambda vector systems  
gt11, gt WES.tB, Charon 4, and plasmid vectors such as  
pBR322, pBR325, pACYC177, pACYC184, pUC8, pUC9, pUC18,

pUC19, pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II SK +/- or KS +/- (see "Stratagene Cloning Systems" Catalog (1993) from Stratagene, La Jolla, Calif, which is hereby incorporated by reference), pQE, pIH821, pGEX, pET series (see F.W. Studier et al., "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes," Gene Expression Technology vol. 185 (1990), which is hereby incorporated by reference) and any derivatives thereof. Recombinant molecules can be introduced into cells via transformation, particularly transduction, conjugation, mobilization, or electroporation. The DNA sequences are cloned into the vector using standard cloning procedures in the art, as described by Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Springs Laboratory, Cold Springs Harbor, New York (1982), which is hereby incorporated by reference.

A variety of host-vector systems may be utilized to express the protein-encoding sequence(s). Primarily, the vector system must be compatible with the host cell used.

Host-vector systems include but are not limited to the following: bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus). The expression elements of these vectors vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

Different genetic signals and processing events control many levels of gene expression (e.g., DNA transcription and messenger RNA (mRNA) translation).

Transcription of DNA is dependent upon the presence of a promoter which is a DNA sequence that directs the binding of RNA polymerase and thereby promoted mRNA synthesis. The DNA sequences of eucaryotic promoters differ from those of procaryotic promoters. Furthermore,

eucaryotic promoters and accompanying genetic signals may not be recognized in or may not function in a procaryotic system, and, further, procaryotic promoters are not recognized and do not function in eucaryotic cells.

5                Similarly, translation of mRNA in procaryotes depends upon the presence of the proper procaryotic signals which differ from those of eucaryotes. Efficient translation of mRNA in procaryotes requires a ribosome binding site called the Shine-Dalgarno (SD) sequence on the  
10 mRNA. This sequence is a short nucleotide sequence of mRNA that is located before the start codon, usually AUG, which encodes the amino-terminal methionine of the protein. The SD sequences are complementary to the 3'- end of the 16S rRNA (ribosomal RNA) and probably promote binding of mRNA to  
15 ribosomes by duplexing with the rRNA to allow correct positioning of the ribosomes. For a review of maximizing gene expression, see Roberts and Lauer, Methods in Enzymology, 68:473 (1979), which is hereby incorporated by reference.

20                The present invention also includes any host cells carrying the recombinatorial substrate. Host cells include bacterial or animal cells, which may be used to maintain or propagate the recombinatorial substrate. Host cells also encompass mammalian cells which have been transformed with  
25 the recombinatorial substrate.

              The present invention also provides for transgenic animals whose somatic and germ cells contain a recombinatorial substrate. One means available for producing a transgenic animal (e.g., a mouse) is as follows:  
30 Female mice are mated, and the resulting fertilized eggs are dissected out of their oviducts. The eggs are stored in an appropriate medium such as M2 medium (Hogan B. et al. Manipulating the Mouse Embryo, A Laboratory Manual, Cold Spring Harbor Laboratory (1986), which is hereby  
35 incorporated by reference). DNA or cDNA encoding gene, minigene or a recombinatorial substrate is purified from a vector (such as plasmids pCEXV-alpha [1a], pCEXV-alpha [1b],

or pCEXV-alpha [1c]) by methods well known in the art. Inducible promoters may be fused with the coding region of the DNA to provide an experimental means to regulate expression of the transgene. Alternatively or in addition, 5 tissue specific regulatory elements may be fused with the coding region to permit tissue-specific expression of the transgene. The DNA, in an appropriately buffered solution, is put into a microinjection needle (which may be made from capillary tubing using a pipet puller), and the egg to be 10 injected is put in a depression slide. The needle is inserted into the pronucleus of the egg, and the DNA solution is injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse (i.e., a mouse stimulated by the appropriate hormones to maintain pregnancy 15 but which is not actually pregnant), where it proceeds to the uterus, implants, and develops to term. As noted below, microinjection is not the only method for inserting DNA into the egg cell, and is used here only for exemplary purposes.

A preferred embodiment of the invention is where 20 the transgenic mammal is selected from a mouse, rat, goat, cow or a pig. The most preferred embodiment is where the mammal is a mouse.

The invention also encompasses a cell line, clone, or tumor derived from a transgenic mammal which contains a 25 recombinatorial substrate. In particular, the invention includes an embryonal stem cell clone which contains a recombinatorial substrate.

Another aspect of the invention is a method of producing a transgenic mammal whose somatic and germ cells 30 contain a recombinatorial substrate. A recombinatorial substrate, which when treated with a recombinase specific to the recombination sites (a) removes the terminator from the DNA molecule, thus permitting transcription of said gene whose expression is to be controlled, or (b) removes said 35 gene whose expression is to be controlled from said DNA molecule, thereby resulting in the loss of function of said gene whose expression is to be controlled, is introduced



into an embryo. The embryo is then transplanted into a pseudopregnant mammal, and the transplanted embryo is allowed to develop to term. A mammal which carries the recombinatorial substrate is identified.

5           The introducing step may be carried out by microinjection or by introducing the DNA molecule into a blastocyst of an embryo or into embryonic stem cells.

          A further embodiment of the invention relates to a method of obtaining mice which are homozygous for the  
10 recombinatorial substrate. Mammals carrying the recombinatorial substrate are interbred and a progeny mammal which carries the recombinatorial substrate on two alleles is identified.

          A preferred embodiment of the invention is where  
15 mammals carrying the recombinatorial substrate are identified by identifying mammals which carry a reporter gene contained in the recombinatorial substrate. The identifying may be carried out by screening for a protein expressed by the reporter gene. For example, by using  
20 antibodies specific to the protein which is expressed. The antibodies may be chemically or radioactively tagged to facilitate detection. The identifying may also be carried out by screening for a phenotype conferred by the reporter gene. An example would be screening for drug resistance or  
25 fluorescence. The identifying may be further carried out by directly screening for the reporter gene or an RNA molecule made by the reporter gene using nucleic acid hybridization techniques.

          Yet another aspect of the invention is a method of  
30 activating the recombinatorial substrate. Recombinase is introduced into a transgenic mammal, carrying the recombinatorial substrate, which when treated with a recombinase specific to the recombination sites (a) removes the terminator from the DNA molecule, thus permitting  
35 transcription of said gene whose expression is to be controlled, or (b) removes said gene whose expression is to be controlled from said DNA molecule, thereby resulting in

the loss of function of said gene whose expression is to be controlled. A mammal which contains an activated recombinatorial substrate is then identified.

The recombinase may be introduced into the cells of the transgenic mammal by a number of delivery methods including chemical or physical methods and via vectors. Preferred chemical and physical methods of delivery include using virosomes, liposomes, naked DNA and particle bombardment, sometimes known as the gene gun.

A preferred approach is to introduce a nucleic acid coding for the recombinase through the use of viral vectors. Viral vectors have the potential of achieving regional gene expression in organotypic slice cultures and in vivo. The preferred vectors include Adenovirus ("Ad") (Akli S. et al., Nat. Genet. 3, 224 (1993); Bajocchi, G., Feldman, S., Crystal, R. and Mastrangeli, A., Nat. Genet., 3, 229 (1993); Davidson, B., Allen E., Kozarsky, J., Wilson, K. and Roessler, B., Nat. Genet., 3, 219, (1993); Le Gal La Salle, G. et al., Science, 259, 988 (1993) which are hereby incorporated by reference), adeno-associated virus ("AAV") (Kaplitt, M. et al., Nat. Genet., 8, 148 (1994a) which is hereby incorporated by reference), and Herpes Simplex Virus ("HSV") (Dobson, A., Margolis, T.P., Sedarati, F., Stevens, J. and Feldman, L., Neuron, 5, 353 (1990); Federoff, H., Geller, A. and Lu, B., Soc. Neurosci Abstr., 16, 353 (1990); Fink, D. et al., Hum. Gene Ther., 4, 11, (1992); Geller, A., Curr. Opin. Gen. Dev., 3, 81 (1993); Geller and Breakefield (1988); Geller, A. and Freese, A. Proc. Natl. Acad. Sci. USA, 87, 1149 (1990); Geschwind, M., Kessler, J., Geller, A. and Federoff, H., Brain Res. Mol. Brain. Res., 27, 327 (1994); Ho, D., Mocarski, E. and Sapolski, R., Proc. Natl. Acad. Sci. USA, 90, 3655 (1993); Kaplitt, M. et al., Mol. Cell. Neurosci., 2, 320 (1991), Proc. Natl. Acad. Sci. USA, 91, 8979 (1994b), which are hereby incorporated by reference).

The most preferred virus is HSV, a 150 kb DNA virus that contains approx. 70 genes (Roizman, B., Virology,

2nd Edn., 1787 (1990), which is hereby incorporated by reference). HSV has a broad host range and appears to be capable of infecting most mammalian differentiated cell types. A number of genes within the wild-type HSV genome are dispensable for its growth in tissue culture. This was initially exploited by Roizman and his colleagues to develop recombinant HSV viruses as vehicles for the transfer of heterologous genes (Roizman, B., Jenkins, F.J., Science, 229, 1208 (1985); Roizman, B. and Sears, A., Virology, 2nd Edn., 1795 (1990), which are hereby incorporated by reference). Subsequently, this recombinant HSV vector approach has been used by a number of investigators and remains a viable gene transfer method that complements the HSV amplicon vector approach to be described (Andersen, J., Garber, D., Meaney, C. and Breakefield, X., Hum. Gene. Ther., 3, 487 (1992); Dobson, A., Margolis, T.P., Sedarati, F., Stevens, J. and Feldman, L., Neuron, 5, 353(1990); Fink, D. et al., Hum. Gene. Ther., 4, 11 (1992), which are hereby incorporated by reference).

The amplicon vector concept arose from analysis of the genomes of defective interfering HSV particles (Spaete, R. and Frenkel, N., Cell, 30, 305 (1982); Stow, N. and McManagle, E., Eucaryotic Viral Vectors, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 199 (1982), which are hereby incorporated by reference) that accumulated in HSV stocks that were passaged at high multiplicities of infection. These genomes were composed of relatively simple reiterations of a subset of DNA sequences from the wild-type HSV genome. Principally, the genomes contained an origin of DNA replication ("ori") and a cleavage/packaging site ("pac") (Spaete, R. and Frenkel, N., Cell, 30, 305 (1982), Spaete, R. and Frenkel, N. Proc. Natl. Acad. Sci. USA, 82, 694 (1985); Stow, N. and McManagle, E., Eucaryotic Viral Vectors, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 199 (1982), which are hereby incorporated by reference). When the ori and pac were cloned into a plasmid it could be replicated and packaged into virions when

transfected into a cell and supplied with HSV replication and virion assembly functions by a superinfecting wild-type virus. Analysis of the genomes in these amplicon stocks indicated that the plasmid-derived sequences were

5 predominantly 150 kb molecules composed of concentrated units of the original plasmid (Spaete, R. and Frenkel, N., Cell, 30, 305 (1982), Spaete, R. and Frenkel, N. Proc. Natl. Acad. Sci. USA, 82, 694 (1985); Stow, N. and McManagle, E., Eucaryotic Viral Vectors, Cold Spring Harbor Laboratory  
10 Press, Cold Spring Harbor, NY, 199 (1982); Vlazny, D., Kwong, A. and Frenkel, N. Proc. Natl. Acad. Sci. USA, 79, 1423 (1982), which are hereby incorporated by reference).

Production of amplicon vectors requires a co-propagated HSV helper virus. Most investigators use helper  
15 viruses that carry mutations in an essential immediate early ("IE") HSV gene, typically IE3 (Geller, A. and Freese, A., Proc. Natl. Acad. Sci. USA, 87, 1149 (1990); Paterson, T. and Everett, R. J. Gen. Virol., 71, 1775 (1990), which are hereby incorporated by reference). Helper virus with a  
20 missense mutation within the IE3 gene have a temperature sensitive phenotype: replication occurs at 34°C but not at 39°C (Preston, C., J. Gen. Virol., 71, 1775 (1979a), Preston C., J. Virol., 29, 275, (1979b), which are hereby incorporated by reference). Deletion of all or part of the  
25 IE3 gene yields viruses that are incapable of growing on normal tissue culture cells but can be grown on cell lines that stably express integrated copies of the IE3 gene. These complementing cell lines have been generated in different cell types and with different transfected gene  
30 segments (DeLuca, N., McCarthy, A. and Schaffer, P., J. Virol., 56, 558 (1985); DeLuca, N. and Schaffer, P., Nucleic Acids Res., 15, 4491, (1987); Paterson, T. and Everett, R. J. Gen. Virol., 71, 1775 (1990), which are hereby incorporated by reference). Helper virus carrying a partial  
35 deletion of the IE3 gene, when grown on a complementing cell line, results in the generation of recombinant wild-type particles. The frequency ( $10^{-5}$ - $10^{-6}$ ) at which such particles

arise appears related to the extent of overlapping homology between the integrated gene and the residual IE3 sequences flanking the deletion in the helper virus genome

(DeLuca, N., McCarthy, A. and Schaffer, P., J. Virol., 56, 558 (1985); DeLuca, N. and Schaffer, P., Nucleic Acids Res., 15, 4491, (1987); Paterson, T. and Everett, R. J. Gen. Virol., 71, 1775 (1990), which are hereby incorporated by reference). Typical amplicon stocks will have titers between 1 and  $10 \times 10^6$  infectious particles of amplicon and between 0.1 and  $5 \times 10^7$  plaque forming units of helper virus.

The typical amplicon vector contains: a plasmid backbone with a Col E1 origin and a drug resistance gene (typically  $\beta$ -lactamase) for growth in *E. coli*; a HSV origin of replication; a cleavage/packaging sequence; and a transcription unit. Initial amplicon vectors were constructed so that a viral promoter, usually an HSV IE promoter (Ho, D., Mocarski, E. and Sapolski, R., Proc. Natl. Acad. Sci. USA, 90, 3655 (1993); Kaplitt, M. et al., Mol. Cell. Neurosci., 2, 320 (1991), which are hereby incorporated by reference) were driving the expression of the gene of interest. Recently some investigators have begun to construct amplicon vectors with multiple genes (Geschwind, M., et al., Hum. Gene Ther., 7, 173 (1996), which is hereby incorporated by reference), regulated promoters (Lu, B. and Federoff, H., Hum. Gen. Ther., 6, 421 (1995), which is hereby incorporated by reference), and cellular promoters (Kaplitt, M., et al., Nat. Genet., 1996, 8, 148 (1994a), which is hereby incorporated by reference) that are helpful in controlling the extent and specificity of gene expression.

Amplicon vectors can efficiently transfer genes into post-mitotic neurons in dissociated cell culture, organotypic slice culture and in the intact brain (Andersen, J., Garber, D., Meaney, C. and Breakefield, X., Hum. Gene Ther., 3, 487 (1992); Bergold P., Casaccia-Bonofil, P., Federoff, H. and Stelzer, A., Soc. Neurosci.

Abstr., 19, 21 (1993a), Bergold, P., Cassaccia-Bonnet, P., Xiu-Liu, Z. and Federoff, H., Proc. Natl. Acad. Sci. USA, 90, 6165 (1993b); Dobson, A., Margolis, T.P., Sedarati, F., Stevens, J. and Feldman, L., Neuron, 5, 353 (1990);

5 Federoff, H., Geller, A. and Lu, B. Soc. Neurosci. Abstr., 16, 353 (1990), Federoff, H., Geschwind, M., Geller, A. and Kessler, J., Proc. Natl. Acad. Sci. USA, 89, 1636 (1992); Fink, D., et al., Hum. Gene Ther., 4, 11 (1992); Geller and Breakefield, 1988; Geller, A. and Freese, A., Proc. Natl.

10 Acad. Sci. USA, 87, 1149 (1990); Geschwind, M., et al., Hum. Gene Ther., 7, 173 (1996); Ho, D., Mocarski, E. and Sapoloski, R., Proc. Natl. Acad. Sci. USA, 90, 3655 (1993); Huang, Q. et al., Exp. Neurol., 115, 303 (1992); Kaplitt, M., et al., Mol. Cell. Neurosci., 2, 320 (1991),

15 Kaplitt, M., et al., Nat. Genet., 8, 148 (1994a); Wolfe, J., Deshmane, S. and Fraser, N., Nat. Genet., 1, 379 (1992), which are hereby incorporated by reference). Most data suggest that *in vivo* expression of transferred genes from amplicon vectors decline within a month (Federoff,

20 unpublished data) although some studies report small numbers of expressing cells at a year (During, M., Naegle, J., O'Malley, K. and Geller, A., Science, 266, 1399 (1994), which is hereby incorporated by reference). In these studies, gene expression was driven by HSV promoters. It is

25 unclear to what extent decreasing expression reflects loss of amplicon genomes, down-regulation of viral promoter transcription or both processes. Studies of viral promoter driven gene expression in different types of vectors suggest that down-regulation is a common problem, thus leading to

30 the speculation that a similar mechanism operates in the amplicon vector.

To satisfy the requirement for spatial and temporal control of gene expression, amplicons need to be capable of producing regional infections of a slice culture

35 and express with a predictable time course. Bath application of virus resulted in widespread gene transfer and expression predominantly in glia on the edge of slice

cultures and little expression in neurons (Casaccia-Bonnefil, P., et al., J. Neurosci. Methods, 50, 341 (1993), which is hereby incorporated by reference). Using a micropipette to deliver nanoliter quantities of virus  
5 directly to regions of the slice culture produced regional infections. Analysis of gene product expression in such cultures showed that it was limited to the microapplication site. With this method, there is a linear relationship between the number of virions applied and the number of  
10 transduced cells (Casaccia-Bonnefil, P., et al., J. Neurosci. Methods, 50, 341 (1993), which is hereby incorporated by reference). Moreover, expression from the HSV IE 4/5 promoter in the amplicons was activated rapidly, within 4 h after virus microapplication (Casaccia-  
15 Bonnefil, P., et al., J. Neurosci. Methods, 50, 341 (1993), which is hereby incorporated by reference).

For this invention any site specific recombinase which can promote recombination in mammals can be used. The preferred recombinases include FLP from yeast and cre. The  
20 most preferred recombinase is cre.

In the examples below, a somatic mosaic approach has been developed for a mouse whereby a dormant germline transgene is activated by the somatic delivery and expression of cre recombinase. Transgenic mice harboring a  
25 recombinatorial substrate, the germline transmitted nerve growth factor excision activation transgene (NGF-XAT), were generated. Somatic delivery of virus vectors expressing cre recombinase into the brain of NGF-XAT mice resulted in regional recombination and activation of the transgene as  
30 demonstrated at the DNA level by PCR and at the protein level by both immunocytochemistry and ELISA. This approach has been exploited to evaluate a behavioral correlation of unilateral NGF mosaicism within the dorsal hippocampal formation. NGF-XAT mice activated by expression of cre  
35 recombinase manifest increased locomotor activity compared to NGF-XAT mice transduced by a control virus expressing *E. coli*  $\beta$ -galactosidase. The following non-limiting examples

show that focally increased expression of NGF in one part of a synaptic network can elicit changes in behavior presumably by altering the overall function of NGF-responsive neural circuitry.

5

## EXAMPLES

### Example 1 - Experimental Protocols

#### **Northern Blot Analysis of Total Brain mRNA From**

10 **NGF XAT Transgenic Lines** The NGF-XAT was excised from pBS and injected into the pronuclei of fertilized eggs, cultured briefly and transferred into the oviduct of pseudopregnant females. Six founders were obtained. Poly A+ RNA was prepared from adult mice of five independent transgenic  
15 lines (#s 30, 2, 25, 3 and 10) by the Fast Track Kit (In Vitrogen, Torrence, CA). Approximately 2 µg of RNA of each were fractionated on a 1.2% agarose gel containing formaldehyde. The gel was blotted to a nylon membrane, prehybridized and then hybridized to a <sup>32</sup>P labeled probe.  
20 The blot was washed under stringent conditions and exposed to film for 14 hours. Because the probe contains approximately 90 bp complementary to the 5' UTR of the endogenous NSE mRNA, it serves also as an internal control for RNA loading.

25 **Nested PCR & Southern Blot Analysis** All PCR reactions were completed using a modified buffer containing 50mM TrisHCl pH 9.0, 16 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.25 mM MgCl<sub>2</sub>, 2% DMSO, and 0.1% Tween-20. One µg of genomic DNA, 1nM of each oligonucleotide, 50 µM dNTP's (Boehringer), and 1 µl of Taq  
30 Polymerase (Perkin-elmer) was added to each 100 µl reaction. Sense primer 1 (5'GCTCGTACGTGCGTCTCCGCCTGCAGCTCTE') and antisense primer 2 (5'AACGCTGTGATCAGAGTGTA 3') was subject to 40 cycles of PCR (Perkin-Elmer 9600) with an annealing temperature of 61°C for 45 seconds and an extension time of  
35 2 minutes at 72°C. One µl of each product was re-amplified using sense primer 3 (5'CATGCGCTCGCTCGGCTCTA 3') and antisense primer 4 (5'GACTCACCGATGCGCGTCCCCTCCGGCTCCA3').



The nested reaction was subjected to 40 cycles of PCR with an annealing temperature of 66°C for 45 seconds and an extension time of 2 minutes at 72°C. Samples were electrophoresed on a 1% agarose gel and visualized by ethidium bromide fluorescence (Fotodyne). PCR product from primer pair 1/2 was electrophoresed on a 1% agarose gel and transferred to nylon membrane (MSI). The blot was prehybridized and then hybridized with <sup>32</sup>P labeled Lox probe. A 123 bp loxP fragment was isolated from SK-lox with Pst 1 and labeled with <sup>32</sup>P dCTP (S.A. 3000 Ci/mmol; Dupont). The blot was washed under stringent conditions and exposed to film for 7 hours.

**Western Blot** 10x10<sup>6</sup> 3T3 cells were plated on a 100 mm plate. The following day, cells were infected with approximately 1 x 10<sup>6</sup> infectious particles of HSVCre or HSVlac for 5 hours. The virus was removed, the cells were washed, and fresh media was added for 24 hours. The following day the media was removed and the cells were washed with 1x PBS and lysed with 0.5 ml of Laemmli buffer for 15 minutes on ice (Laemmli, U. Nature 227, 680 (1970), which is hereby incorporated by reference). 250 ng of lysate per well was run on a 4-20% gradient gel (Bio-Rad) with 15 µl of Low Molecular weight markers (Amersham) as size standards. The gel was run for 35 minutes at 100 volts. Transfer of the gel onto a nitrocellulose filter (Protran BA 83, S&S) was completed overnight. A 1/10,000 dilution of Cre antibody (generously provided by DuPont, Inc.) in 5% milk and PBS was added to the blot and incubated overnight at 4°C. The blot was developed following the ECL western blotting protocol using a secondary antibody recommended by the manufacturer (Amersham). The blot was exposed to film (XAR, Kodak) for 10-20 seconds and then developed.

**Stereotaxic Injections** Mice were anesthetized with 3% halothane in 70% N<sub>2</sub>O and 30% O<sub>2</sub> in the induction anesthesia chamber and maintained at 2% halothane during stereotaxic intracerebral injections. After positioning in a Kopf stereotaxic apparatus, the skull was exposed via a midline incision and burr holes were drilled. The coordinates, relative to Bregma, for NGF immunocytochemistry were -2.0 mm, lateral 2.5 mm, and deep 1.8 mm; and those for the behavioral studies were -2.0 mm, lateral 1.5 mm and deep 1.5 mm. A 33 GA needle was gradually advanced to the desired depth and 2-3  $\mu$ l (approximately 10<sup>5</sup> infectious particles) were slowly infused over the time course of 5 minutes. Four days later, half of the mice were killed by halothane overdose for X-gal staining. Fourteen days later the remaining mice were prepared for  $\beta$ -NGF immunocytochemistry.

**X-gal Staining** Frozen sections (20  $\mu$ m) from HSVcrelac and HSVlac injected animals were mounted on subbed slides and stored at -20°C. Sections were post-fixed for five minutes with 1% glutaraldehyde. Slides were washed three times with 1x PBS and submerged in a solution containing: 5 mM potassium Ferricyanide, 5 mM potassium ferrocyanide, 0.02% NP-40, 0.01% sodium deoxycholate, 2 mM MgCl<sub>2</sub> and 1  $\mu$ g of X-Gal (Gibco). Slides were incubated overnight at 37°C and counterstained with thionin for three minutes. After staining sections were dehydrated and coverslipped as described in the  $\beta$ -NGF immunostaining method.

**$\beta$ -NGF Immunocytochemistry** Adult mice were anesthetized with 3.0% halothane in 70% N<sub>2</sub>O and 30% O<sub>2</sub> using a Fluotec 3 vaporizer (Colonial Medica, Amherst, NH). A 24 GA angiocatheter (Angiocath, Deseret Medical Inc., Sandy, UT) was placed into the apex of the left ventricle and intracardiac perfusion was initiated with 10 ml of heparinized saline (5,000 U/liter saline) followed by 20 ml of chilled solution of 2% paraformaldehyde in 0.075 M phosphate buffer (pH=7.5, Mallinckrodt UN 2213) in 0.2%

parabenzquinone. Brains were extracted and postfixed in paraformaldehyde-parabenzquinone solution for additional 2-3 hrs at 4C. Following that, the brains were placed into 10 ml of a cryoprotective solution of ethylene glycol (30%) and sucrose (20%) in 0.1 M phosphate buffer (pH=7.5) for 24 hrs. Forty micron serial sections were cut on powdered dry ice using an American Optical sliding microtome and stored in phosphate buffer containing 0.06% azide at +4°C until processed for  $\beta$ -NGF immunocytochemistry. Representative coronal sections of the mouse brains were placed into Costar net wells (VWR) and incubated for 10 min in 0.1 M Tris buffered saline (pH=7.6) followed by 2 consecutive 10 minutes washes in TBS with 0.25% Triton (T-8787, Sigma). Blocking was performed for 60 min in a solution of 2% crystalline grade bovine serum albumin (A-8022, Sigma) and 5% normal goat serum in TBS followed by a brief 5 min wash in 0.25% Triton solution of TBS. Staining with rabbit polyclonal (affinity purified) antibodies to mouse  $\beta$ -NGF (1:1000 dilution or 1  $\mu$ g/ml) was performed for 60-64 hrs as recommended by Conner et al, Muir, D., Varion, S., Hagg, T. and Manthorpe, M. J Comp Neurol 319, 454 (1992) (which is hereby incorporated by reference) at +4C. Bound antibodies were detected by incubating sections in 1.5  $\mu$ g/ml of biotinylated goat anti-rabbit antibody (Vectastain Elite, rabbit IgG ABC kit, PK-6101, Vector labs, Burlingame, CA) in a 0.25% triton solution of TBS with 0.5% goat serum for 3 hrs at room temperature. Sections were then incubated at room temperature for 90 min in the avidin-biotin-peroxidase reagent according to the manufacturer's recommendations. After consecutive 10 minutes rinses in TBS and Tris-HCl the sections were placed for 7 min into a filtered solution of 0.04% diaminobenzidine (D-5637, Sigma), 0.06% nickel chloride (N 5756, Sigma) and 0.06% hydrogen peroxide in 0.1 M Tris HCl buffer (pH 7.4). Sections were then rinsed in phosphate buffer, mounted with a fine paintbrush on superfrost coated slides (Fisher), dehydrated in 95% and

100% alcohols, incubated in histological grade xylene, coverslipped with permount, and left to dry for 24 hrs.

**NGF ELISA** Brain regions containing the injection site were surgically isolated from the animals injected with either HSVcrelac and HSVlac. They were extracted in buffer containing PBS, 0.4 M NaCl, 0.05% Tween 20, 0.5% BSA, 0.1 mM benzethonium chloride, 1 mM PMSF, and 3.5 µg/ml of aprotinin in a 1:1 weight/volume proportion. The samples were homogenized in a micro glass tissue grinder (Wheaton).

Samples were then subjected to five rounds of cup sonication at 40% output power. Homogenates were centrifuged at 16,000 x g at 4°C for 60 minutes. Supernatants were diluted serially with a buffer containing 50 mM Tris-HCl, 200 mM NaCl, 10 mM CaCl<sub>2</sub>, 1% BSA, 0.1% Triton X-100 and 0.1% sodium azide and added to High Binding ELISA plates (Costar) that are prepared as follows. ELISA plates were coated with a 1 µg/ml solution of anti-rhNGF (generously supplied by Dr. Dominick Sinicropi), incubated for 2 hours at 37°C and then blocked for 1 hour at 37°C. Supernatants and murine NGF standards were added and incubated overnight at 4°C. Anti-NGF-βgal conjugate (Boehringer Mannheim) was added for four hours at 37°C and the assay developed according to the manufacturers protocol (Boehringer Mannheim). Samples were read at 595nm (Bio-Rad) and a standard curve was derived from murine NGF controls. Brain samples for both HSVcrelac and HSVlac injected animals were measured against the standard curve.

**In Situ Hybridization** *In situ* hybridization was performed on 12 µm coronal sections using the Boehringer Mannheim Digoxigenin labeling and detection system. Oligonucleotide probes (45 mer's) complementary to hGH Exon II were 3'-end labeled for 10 minutes and used in a 1:50 dilution. Frozen sections were brought to room temperature and fixed in 4% paraformaldehyde for 15 minutes. After fixation the sections were washed once in 1x PBS and incubated at room temperature in 2x SSC. Sections were then rinsed two times in 1X PBS and digested with proteinase K

(20  $\mu\text{g/ml}$ ) at 37°C for 20 minutes. Sections were postfixed in 4% paraformaldehyde for 5 minutes and then washed in PBS. Prior to prehybridization the sections were acetylated to decrease nonspecific binding of probe. Sections were  
5 incubated in triethanolamine and 0.5% acetic anhydride for 10 minutes followed by a 10 minutes wash in 2x SSC. Prehybridization buffer consisting of: 50% deionized formamide, 4x SSC, 1x Denhart's, 10% Dextran sulfate, 30  $\mu\text{g/ml}$  salmon sperm DNA and 25  $\mu\text{g/ml}$  yeast tRNA, was added to  
10 the sections and allowed to incubate at room temperature for one hour. After prehybridization sections were rinsed briefly in 2x SSC. Probe was denatured for 5 minutes at 95°C and added to prehybridization solution in a 1:50 ratio and incubated overnight at 37°C. Sections were washed with  
15 increasingly stringent washes (SSC: 2x, 1x, 0.5x) first at room temperature and 37°C over a period of 3 hours. Anti-digoxigenin was added in a 1:500 dilution to the sections in the presence of 1% sheep serum and 0.003% Triton X-100 and incubated at room temperature for 4 hours. After several  
20 Tris-HCl washes color detection solution was added (NBT & X-Phosphate) to the slides and they were developed overnight.

**Locomotor Activity** Horizontal and vertical locomotor activity measurements were made in an automated  
25 device based on infra-red beam breaks (Opto-Varimex Minor, Columbus Instruments International Corporation) at 5 minute intervals over the course of a 90 minutes experimental session on a single day. Each beam was separated by 25.4 mm. with the width of each beam being 3 mm in diameter.  
30 Interruption of any horizontal or vertical beam generated an electrical impulse that was collected using the Opto-M data software. Horizontal beams registered only when the mouse moved in the horizontal plane of the test cage, and jumping and rearing were registered by the vertical beam.  
35 Statistical analyses were carried out using 2 way repeated measures analyses of variance with transgene as a between

group variable and time interval as a within group variable (SuperANOVA, Abacus Concepts, Inc.).

5     Example 2 -     Design of a Germline Transmitted  
                  Recombinatorial Substrate

          A germline transmitted recombinatorial substrate, the NGF excisional activation transgene (NGF-XAT), was designed to synthesize a neuron-specific non-coding  
10 transcript that upon activation by somatically expressed cre recombinase an inactivating cassette is excised allowing for NGF expression. The NGF-XAT (Figure 2A) is driven by the 1.8 kb neuron specific enolase ("NSE") promoter, contains a loxP bounded mutated fragment of the human growth hormone  
15 ("hGH") gene fused to the  $\beta$ -globin transcription terminator, the NGF mini-gene, and a IRES initiated neomycin phosphotransferase gene ("IRESNpt"), and 3' flanking sequence from hGH gene.

20     Example 3 -     Demonstration of Recombination Driven  
                  Excision in *E. coli*

          To demonstrate the efficiency and fidelity of excision of the inactivating cassette, the NGF-XAT was  
25 introduced into bacteria constitutively expressing the cre recombinase (Figures 2B and 2C, lanes 1-9) and also control bacteria lacking the recombinase (lanes 11-22). Plasmid miniprep DNA from independent transformants from both strains were prepared, digested with BamH1, size  
30 fractionated on an agarose gel, and Southern blotted. Southern blot analysis of the independent bacteria plasmids revealed highly efficient removal: all colonies lacked the restriction fragment corresponding to the inactivating cassette. A BamH1 digest of control NGF-XAT DNA was run in  
35 lane 10. The results are shown in Figure 2B. In Figure 2B, the blot was hybridized with probe (see Figure 2A). Only plasmids prepared from bacteria lacking cre recombinase contained this fragment (Fragment B); all plasmids from bacteria expressing cre recombinase had deleted this

fragment. In Figure 2C the blot was hybridized to probes 2 and 3, demonstrating that the plasmids from both types of bacteria liberated the expected fragments (Fragments A, A1, and C). Fragment A1 is slightly larger than Fragment A because recombination creates a new 3' BamHI site. Direct sequencing of the "collapsed" construct indicated removal of the cassette. By contrast, the intact NGF-XAT was recovered from all bacteria that did not express cre recombinase.

10 Example 4 - Generation of Transgenic Mice

The NGF-XAT was excised from pBS, injected into the pronuclei of fertilized eggs, cultured briefly, and transferred into the oviduct of pseudopregnant females. Six founders were obtained. Poly A+RNA was prepared from adult mice of five independent transgenic lines (#s 30, 2, 25, 3 and 10) by the Fast Track Kit (In Vitrogen, Torrence, CA). Approximately 2  $\mu$ g of RNA of each were fractionated on a 1.2% agarose gel containing formaldehyde. The gel was blotted to a nylon membrane, prehybridized and then hybridized to a  $^{32}$ P labeled probe (Northern Probe 1 in Figure 3A). The blot was washed under stringent conditions and exposed to film for 14 hours (Figure 3B). Because the probe contains approximately 90 bp complementary to the 5' UTR of the endogenous NSE mRNA, RNA loading was internally controlled.

Of the five independent lines of transgenic mice which were generated, two expressed the predicted truncated, inactive transcript (The location of the smaller NGF XAT transcripts in expressing lines #30 and 10 are shown in Figure 3A). Of the two lines, line #30 produced larger amounts of transcript, that corresponds after correction for the length of probe complementarity to approximately 15% of the amount of the native NSE mRNA (Figure 3B). *In situ* hybridization studies using an antisense probe complementary to the truncated, inactive transcript revealed extensive labeling in a neuronal pattern (Figures 4A and 4B), whereas

transgenic sections probed with a sense probe were negative (Figure 4C). Additionally, no labeling above background was observed with antisense probe when hybridized to sections from non-transgenic mice. Rehybrization of the blot to Northern Probe 2 (Figure 4A) revealed no NGF transgene transcripts, confirming efficient transcription termination.

Example 5 - Promotion of Recombination *in vivo* by Somatic Delivery of cre Recombinase

10

To determine whether somatic delivery of cre recombinase could promote recombination *in vivo* we generated several Herpes Simplex Amplicon (HSV) virus vectors that express recombinase (Figure 5A; HSVcre). HSVcre was constructed by cloning cre (Gu, H., Zou, Y.-R. and Rajewsky, K., Cell., 73, 1155 (1993)) into HSVPrPUC (Battleman, D., Geller, A. and Chao, M., J Neurosci., 13(3), 941 (1993), Bergold, P., Cassaccia-Bonnet, P., Xiu-Liu, Z. and Federoff, H., Proc. Natl. Acad. Sci. USA, 90, 6165 (1993), which are hereby incorporated by reference). The gene, HSVcre, was packaged into virus (Geschwind, M., Lu, B. and Federoff, H., Providing pharmacological access to the brain; A volume of Methods in Neurosciences, Conn ed. 1994, which is hereby incorporated by reference) and used to infect NIH3T3 cells to detect recombinase expression. 30 hours later, cells were harvested and protein extracts prepared and subjected to western blot analysis (Hassankhani, A., Steinhilber, M., Soonpaa, M., Katz, E., Taylor, D., Rozenthal, A., Factor, S., Steinberg, J., Field, L. and Federoff, H., Dev Biol., 169, 309 (1995), which is hereby incorporated by reference). Protein extracts from *E.coli* expressing cre recombinase (Figure 5B, lane 1) not expressing recombinase (lane 2), HSVcre infected 3T3 cells (lane 3), HSVlac (expresses  $\beta$ -galactosidase) infected 3T3 cells (lane 4), and uninfected 3T3 cells (lane 5) were fractionated by SDS-PAGE, blotted, and probed with a rabbit polyclonal antibody directed against cre



recombinase. A cre-specific band (arrow) is observed only in bacteria expressing the recombinase (lane 1) and HSVcre infected 3T3 cells (lane 3). (Figure 5C) The NGF-XAT construct is depicted before recombination (Top) and after recombination (Bottom) and the location of PCR primers are shown. (Figure 5D) The PCR was used to monitor Cre recombination *in vivo*.

Focal delivery of HSVcre but not control virus HSVlac (expresses  $\beta$ -galactosidase) into the dorsal hippocampus of line #30 mice resulted in excision of the inactivating cassette from the NGF-XAT germline construct (Figure 5C) as demonstrated as direct PCR-Southern blot analysis (Figure 5D) and nested PCR (Figure 5E). Line #30 NGF-XAT mice were infected with HSVcre and HSVlac and brain regions from the injection sites and other noninjected regions harvested. DNA was prepared and subjected to PCR with the primers shown in C (Battleman, D., Geller, A. and Chao, M., J Neurosci., 13(3), 941 (1993), which is hereby incorporated by reference). The PCR products were blotted, hybridized with a  $^{32}\text{P}$  labeled loxP probe, washed stringently and exposed to film as described. A full length 3,100 bp product was observed from a plasmid template containing the non-recombined NGF-XAT (Figure 5D; lane 1). A collapsed PCR product of 785 bp (arrow) was seen only in brain regions injected with HSVcre virus (Figure 5D; lane 3) that is the same size as that product derived by PCR on a collapsed template produced by bacteria expressing cre recombinase (Figure 5D; lane 2). No collapsed PCR fragment was observed in DNA from a HSVlac infected line #30 animal (Figure 5D; lane 4) or an uninjected animal (Figure 5D; lane 5). Full length product was not efficiently produced from transgenic line #30 animals, although PCR amplification with HF 37/47 for the NGF minigene (Federoff, H., Geschwind, M., Geller, A. and Kessler, J., Natl. Acad. Sci. USA, 89, 1636 (1992), Hassankhani, A., Steinhelper, M., Soonpaa, M., Katz, E., Taylor, D., Rozental, A., Factor, S., Steinberg, J., Field, L. and Federoff, H., Dev Biol., 169,

309 (1995)) was positive. (Figure 5E) Nested PCR analysis: DNAs extracted from virus infected animals, were amplified with Primers 1/2, reamplified with Primers 3/4, and fractionated on and ethidium bromide agarose gel

5 (Battleman, D., Geller, A. and Chao, M., J Neurosci., 13(3), 941 (1993), which are hereby incorporated by reference). A full length 2,700 bp fragment was observed from a plasmid template containing the non-recombined NGF-XAT (Figure 5E; lane 1). A collapsed PCR produce of 318 bp (arrow) was  
10 observed only in brain regions injected with HSVcre virus (Figure 5E; lane 3) that is the same size as that product derived by PCR on a collapsed template produced by bacteria expressing cre recombinase (Figure 5E; lane 2). No collapsed PCR fragment was observed in DNA from a HSVlac-  
15 infected line #30 animal (Figure 5E; lane 4) or an uninjected animal (Figure 5E; lane 5). Full length product was not efficiently produced from transgenic line #30 animals, although PCR amplification with HF 37/47 for the NGF minigene (Federoff, H., Geschwind, M., Geller, A. and  
20 Kessler, J., Natl. Acad. Sci. USA, 89, 1636 (1992), Hassankhani, A., Steinhelper, M., Soonpaa, M., Katz, E., Taylor, D., Rozental, A., Factor, S., Steinberg, J., Field, L. and Federoff, H., Dev Biol., 169, 309 (1995), which are hereby incorporated by reference) was positive.

25 Example 6 - Focal Delivery of cre Expressing HSV Vector

This example demonstrates that the somatic expression of recombinase activated the NGF-XAT construct  
30 for required analysis of the transgene product, NGF, by immunocytochemistry. Line #30 mice were stereotaxically injected unilaterally with either HSVlac or HSVcrelac (expressing both nuclear localized Cre and  $\beta$ -galactosidase) virus (approximately  $2 \times 10^5$  infectious particles). Some  
35 animals were analyzed after 4 days to localize expression of  $\beta$ -galactosidase by X-gal histochemistry to confirm localization of vector expression (Geschwind, M., Lu, B. and

Federoff, H., Providing pharmacological access to the brain;  
A volume of Methods in Neurosciences, Conn ed. 1994, which  
is hereby incorporated by reference). Sections from both  
HSVlac and HSVcrelac showed robust  $\beta$ -galactosidase  
5 expression confined to the virus injection site. A separate  
group of animals were injected with both viruses and  
analyzed 14 days later by NGF immunocytochemistry. HSVlac  
injected animals (Figure 6C) showed no alteration in the  
intensity or pattern of immunoreactivity on the injected  
10 side (arrow) compared with the control side or uninjected  
animals (Figures 6A, 6B). HSVcrelac infected animals (6D)  
demonstrated a marked increase in the distribution of NGF  
immunoreactivity that was localized to the virus injection  
region (arrow) and not anterior or posterior to the virus  
15 injection site (data not shown).

Example 7 - Quantification of NGF Immunoreactivity by  
ELISA

20 Direct quantification of NGF immunoreactivity  
was performed by ELISA 14 days after virus injection.  
Line #30 mice were stereotaxically injected unilaterally  
with either HSVlac or HSVcrelac (expressing both nuclear  
localized Cre and  $\beta$ -galactosidase) virus (approximately  $2 \times$   
25  $10^5$  infectious particles), and sacrificed after 14 days.  
Tissue blocks containing the injection sites were harvested,  
homogenized, and analyzed by ELISA. A large and significant  
(\* $P < 0.001$ , t-test) increase in NGF content was noted in the  
HSVcrelac (Figure 7, hatched bar,  $n=5$ ) compared to the  
30 HSVlac (open bar,  $n=3$ ) injected animals. HSVcrelac injected  
animals manifest an approximately 15 - fold increase in NGF  
concentration within the injected region as compared to  
HSVlac and uninjected control animals.

Example 8 - Increased Spontaneous activity in mice with a cre-activated NGF-XAT transgene

Cre-mediated activation of the NGF-XAT gene within  
5 a terminal innervation field of the hippocampus was used to  
determine whether increased focal NGF function would alter  
spontaneous behavior. NGF-XAT mice were injected  
stereotactically into the dorsal hippocampus with either  
10 HSVcrelac or control vector HSVlac. Within two weeks, post  
injection animals injected with HSVcrelac were first noted  
to be more active. At between 4 and 6 months post injection  
behavioral studies were performed.

Horizontal and vertical locomotor activity  
measurements were made in an automated device based on  
15 infra-red beam breaks (Octo-Varimex Minor, Columbus  
Instruments International Corporation) at 5 minute intervals  
over the course of a 90 minutes experimental session on a  
single day. Each beam was separated by 25.4 mm. with the  
width of each beam being 3 mm in diameter. Interruption of  
20 any horizontal or vertical beam generated an electrical  
impulse that was collected using the Octo-M data software.  
Horizontal beams registered only when the mouse moved in the  
horizontal plane of the test cage, and jumping and rearing  
were registered by the vertical beam. Statistical analyses  
25 were carried out using 2 way repeated measure analyses of  
variance with transgene as a between group variable and time  
interval as a within group variable (SuperANOVA, Abacus  
Concepts, Inc.).

Line #30 mice were stereotaxically injected  
30 unilaterally with either HSVlac or HSVcrelac (expressing  
both nuclear localized cre and  $\beta$ -galactosidase) virus  
(approximately  $2 \times 10^5$  infectious particles) and after 4 to  
6 months animals were studied behaviorally. The results are  
summarized in Figure 8. Total horizontal (top) and vertical  
35 (bottom) activity counts during each 5 minute interval over  
a 90 minutes experimental session for HSVlac (filled  
circles) and HSVcrelac (open circles) mice. Each data point

represents the group mean  $\pm$  S.E. value and 8 mice for a 5 minutes interval. The groups were age-matched and comprised of 6 males and 2 females.

Horizontal activity levels of HSVcrelac and HSVlac mice were generally equivalent over the first 30 min of the session, but levels subsequently declined less rapidly in HSVcrelac than HSVlac mice over the next 60 min, resulting in significantly higher horizontal activity levels in HSVcrelac mice (main effect of transgene activation,  $F=8.177$ ,  $df=1, 14$ ,  $p=.012$ ; transgene activation by time interaction,  $F=2.58$ ,  $df=17, 238$ ,  $p=.0008$ ). Vertical activity levels were likewise significantly increased in HSVcrelac mice (main effect of transgene activation,  $F=33.29$ ,  $df=1, 14$ ,  $p=.0001$ ) with the increase sustained over the course of the 90 min session (time by transgene activation interaction,  $F=1.57$ ,  $df=17, 238$ ,  $p=.072$ ). Differences in vertical activity were particularly notable, with peak increases in vertical activity in HSVcrelac mice (time points 7-9) on the order of 1139-1382% of HSVlac values. These increases in activity occurred despite the slightly larger body weights of HSVcrelac mice (HSVcrelac  $36.17 \pm$  S.E. 1.91; HSVlac  $31.25 \pm$  S.E. 1.81;  $p=0.41$ ), which would normally be predicted to decrease motor activity levels. Although the neuroanatomic and physiologic changes underlying this behavioral differences are not yet known, the initiating event is focally increased NGF expression within the hippocampal formation.

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.